JOURNAL OF VIROLOGY, Apr. 2006, p. 3386–3394 Vol. 80, No. 7
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.7.3386–3394.2006

Differential Utilization of CD134 as a Functional Receptor by Diverse Strains of Feline Immunodeficiency Virus

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Received 29 July 2005/Accepted 4 January 2006

The feline homologue of CD134 (fCD134) is the primary binding receptor for feline immunodeficiency virus (FIV), targeting the virus preferentially to activated $CD4^+$ helper T cells. However, with disease progression, the cell tropism of FIV broadens such that B cells and monocytes/macrophages become significant reservoirs of proviral DNA, suggesting that receptor utilization may alter with disease progression. We examined the receptor utilization of diverse strains of FIV and found that all strains tested utilized CD134 as the primary receptor. Using chimeric feline × human CD134 receptors, the primary determinant of receptor function was mapped to the first cysteine-rich domain (CRD1) of fCD134. For the PPR and B2542 strains, the replacement of CDR1 of fCD134 (amino acids 1 to 64) with human CD134 (hCD134) alone was sufficient to confer nearly optimal receptor function. However, evidence of differential utilization of CD134 was revealed, since strains GL8, CPGammer (CPG41), TM2, 0827, and NCSU1 required determinants in the region spanning amino acids 65 to 85, indicating that these strains may require a more stringent interaction for infection to proceed.

The initial event in the process of viral entry into a target cell is the interaction between the virus and its cellular receptor, and the specificity of this interaction determines both the cell tropism and the pathogenicity of the virus. The primary receptor for human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) is CD4, targeting the virus to helper T cells and resulting in progressive depletion of these cells and the eventual development of AIDS (46). However, CD4 expression alone is insufficient to confer susceptibility to infection with HIV, which also depends on the expression of coreceptors, principally the chemokine receptors CXCR4 and CCR5 (3). The virus attaches via a high-affinity interaction with CD4, resulting in a conformational change in the envelope glycoprotein (Env) and exposing the binding site for the chemokine receptor (30). This then triggers a further conformational change that exposes the fusion domain of the viral transmembrane protein gp41 and enables fusion of the viral and cellular membranes (17).

Feline immunodeficiency virus (FIV) is unique among the nonprimate lentiviruses because in its natural host species, the domestic cat, it induces a disease similar to AIDS, which is characterized by a progressive depletion of $CD4^+$ T lymphocytes (39). In chronic infection, an immunodeficiency syndrome develops that is characterized by wasting, neurological manifestations, chronic stomatitis and gingivitis, and an increased incidence of lymphoma (38). In contrast, the ungulate lentiviruses induce diseases reminiscent of chronic inflammatory conditions. However, CD4 is not the primary receptor for FIV, as the primary receptor was recently revealed to be CD134 (OX40) (48), a member of the tumor necrosis factor receptor/nerve growth factor receptor superfamily. Primary isolates of FIV use CD134 as the binding receptor in conjunction with the chemokine receptor CXCR4 as a cofactor for infection (45, 48, 60). FIV binds specifically to CD134-expressing cells (48), the FIV Env interacts directly with CD134 (13), and pretreatment of virus with soluble CD134 facilitates infection of $CD134^-$ $CXCR4^+$ cells (12).

CCR5 appears to be the coreceptor utilized by the majority of HIV strains early in infection, and the usage of CXCR4 as a coreceptor is more frequent with disease progression (10, 47). In contrast, FIV appears to use CXCR4 alone as its sole coreceptor for infection (19, 45, 61), and yet, with disease progression, the viral cell tropism expands (11, 20), suggesting an alteration in receptor utilization. Previous studies have demonstrated that upregulation of endogenous CXCR4 can increase the susceptibility to infection with FIV (59) and that overexpression of exogenous human CXCR4 on a target cell may overcome the requirement for the expression of CD134 (14). Thus, it is possible that with disease progression, FIV may either lose its dependence on an interaction with CD134 or interact more efficiently with CXCR4.

In this study, we investigate the use of CD134 as a functional receptor by diverse strains of FIV. As a first step towards understanding the role of CD134 in the pathogenesis of FIV infection, we examine the receptor utilization of a panel of FIV isolates of diverse subtypes and geographic origins. We define the region of CD134 that confers functional FIV receptor activity and present evidence for the differential usage of CD134 as a viral receptor by diverse strains of FIV.

MATERIALS AND METHODS

Cells and viruses. MYA-1 (33), MCC (7), and NSO cells were cultured in RPMI 1640 medium. 293T and HeLa cells were maintained in Dulbecco's modification of Eagle's medium. All media were supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.11 mg/ml sodium pyruvate, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The medium for MYA-1 cells was supplemented with conditioned medium from a murine cell line (L2.3) transfected with a human

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interleukin-2 (IL-2) expression construct (equivalent to 100 U/ml of recombinant human IL-2) and 50 μM 2-mercaptoethanol. All media and supplements were obtained from Invitrogen Life Technologies Ltd. (Paisley, United Kingdom). Cell lines expressing CD134 and the chimeric constructs were maintained in G418 (Invitrogen, Paisley, United Kingdom). The canine lymphocytic leukemia (CLL) cell line was generated by prolonged in vitro culture of peripheral blood mononuclear cells; the established line is $CD3^+$ $CD4^-$ $CD8^-$.

The B2542 (subtype B) (15) and CPGammer (subtype C) (16) isolates of FIV were obtained from E. Hoover and S. Vandewoude. Molecular clones of TM219 (32) and NCSU1 (JSY3) (63) were obtained from T. Miyazawa and W. Tompkins, respectively. PPR (41) Env (encoded by plasmid pΔ11 [37]) was obtained from G. Pancino. pFIV-PPR was obtained from John Elder, NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. GL8 (24), 0827, 0425, and 1419 are subtype A field isolates of FIV from the United Kingdom.

Antibodies and flow cytometry. Anti-human CD134 (BerACT35) was obtained from Alexis Corporation. Anti-human CXCR4 (44701) was obtained from R&D Systems, Abingdon, Oxford, United Kingdom. Cells to be processed for flow cytometry were resuspended in phosphate-buffered saline supplemented with 1.0% (wt/vol) bovine serum albumin and 0.1% (wt/vol) sodium azide (PBA). Cells were incubated with 1 μg of primary antibody for 30 min at 4°C and then washed twice with PBA by centrifugation at 1,000 rpm for 5 min. Bound primary antibody was detected with an appropriate anti-mouse immunoglobulin G secondary antibody (Serotec, Oxford, United Kingdom) corresponding to the isotype of the primary antibody and conjugated to either fluorescein isothiocyanate or R-phycoerythrin (RPE). Cells were incubated with secondary antibody for 30 min at 4°C, washed twice with PBA by centrifugation at 1,000 rpm for 5 min, and resuspended in 1 ml of PBA for analysis. All samples were analyzed on a Beckman Coulter EPICS MCS-XL flow cytometer, with 10,000 events being collected for each sample in list mode. Data were analyzed using EXPO 32 ADC analysis (Advanced Cytometry Systems).

Construction of feline × human CD134 chimeras. Previously, we demonstrated that feline, but not human, CD134 supported infection with FIV (48). The amino acid sequences of feline and human CD134 (fCD134 and hCD134, respectively) were aligned using the Clustal W algorithm (55), enabling the identification of nonconserved amino acids, many of which were localized to the N-terminal (extracellular) region of the molecule. The predicted three-dimensional structure of feline CD134 was generated using Swiss-Model (40) in first-approach mode, and images were then manipulated using Swiss-Pdb Viewer v3.7b2 (Glaxo Wellcome Experimental Research). The three-dimensional structure prediction enabled the identification of nonconserved amino acids that were either within or in close proximity to the N-terminal cysteine-rich domains (CRDs). A strategy was therefore devised that would enable the generation of feline × human chimeric CD134 molecules by creating chimeric junctions in conserved regions of amino acid sequence, preserving the sequence and thus being less likely to affect the tertiary structure of the chimeric protein. To facilitate the construction of these chimeras, NruI and BsrGI restriction sites were introduced into the feline and human CD134 cDNA clones by PCRs with the primers 5-CCAATACCCTACCTCAGCGCTACGTCG-3′ (NruI site is underlined) and 5′-GGCACGTTCGGGACATGT-3′ (BsrGI site is underlined). These restriction sites created chimeric junctions between residues 56 and 57 and residues 85 and 86 of feline CD134. Thus, the chimera FHF represents feline $CD134_{1\text{-}56}$ × human $CD134_{57\text{-}85}$ × feline $CD134_{86\text{-}270}$. Additional chimeras were prepared in which amino acids 47 to 56 of human CD134 were introduced into the FFH chimera, generating (FH)FH, and in which amino acids 65 to 85 of feline CD134 were introduced into the FHH chimera, generating F(FH)H. The nucleic acid sequence of each of the chimeric CD134 molecules was confirmed by cycle sequencing on an Applied Biosystems 9700 thermal cycler, using a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems), followed by analysis on an Applied Biosystems 3700 genetic analyzer. cDNAs were subcloned into the retroviral vectors pDONAI (Takara, Tokyo, Japan) and pCNC-MCS (25).

In order to generate CD134-enhanced green fluorescent protein (CD134-EGFP) gene fusions, the coding sequences for the CD134 molecules and CD134 chimeras were reamplified with the primers 5′-TTGGTACCATGAGGGTGGT TGTGGGGGCT-3′ (KpnI site is underlined) and 5′-ATCCCGGGCGA TCTT GGCCAGGGTGGAGTT-3′ (SmaI site is underlined) and cloned into pEGFPN1 (BD Biosciences Clontech, Cowley, Oxford, United Kingdom). The resulting gene fusions were then subcloned into pDONAI or pCNC-MCS (25). The nucleic acid sequences of all gene fusions were confirmed as described above.

HIV pseudotype assays. The FIV *env* gene expression constructs GL8, 0827, 0425, 1419, PPR, and TM2 have been described previously (48). The B2542 and CPGammer *env* genes were amplified by PCR from DNA prepared from infected MYA-1 cells and cloned directly into VR1012 (Vical Inc., San Diego, CA). Five micrograms of VR1012-*env*, NCSU1*env*-cDNA3 (21), or pCI-VSV-G and 7.5 μg of pNL4-3-Luc-E^-R^- were cotransfected into HEK-293T cells, using the calcium phosphate coprecipitation technique essentially as described previously (23). Culture supernatants were collected at 48 h posttransfection, filtered with a 0.45-μm filter, and frozen at −70°C until required. Adherent target cell lines were seeded at 1×10^4 cells per well in a CulturPlate-96 assay plate (Perkin-Elmer, Life and Analytical Sciences, Beaconsfield, United Kingdom) and cultured overnight, while suspension target cell lines were seeded at 5×10^4 cells per well and used immediately. The cells were then infected with 50 μl HIV (FIV) luciferase pseudotypes and cultured for 72 h, and luciferase activity was quantified by the addition of 50 μl of Steadylite HTS (Perkin-Elmer) luciferase substrate and measurement by single photon counting on a MicroBeta luminometer (Perkin-Elmer).

Growth of FIV in vitro. The growth of FIV in vitro was assessed in CLL cells transduced with each of the chimeric CD134 constructs. Cells (1×10^6) were infected with 0.45-μm-filtered culture supernatant from FIV-infected MYA-1 cells containing approximately 10,000 50% tissue culture infective doses (titrated on MYA-1 cells) of virus for 2 h at 37°C. The cells were then pelleted by centrifugation at 1,000 rpm, the medium was aspirated, and the cells were washed twice with phosphate-buffered saline. The cells were then resuspended in culture medium and plated in 12-well culture plates. Supernatants were collected every 3 days and assayed for reverse transcriptase (RT) activity using a Lenti-RT nonisotopic RT assay kit (Cavidi Technology, Uppsala, Sweden). RT values were then calculated relative to that for a purified HIV type 1 (HIV-1) RT standard.

RESULTS

BerACT35 recognizes feline CD134. Previous studies have used the anti-human CD134 monoclonal antibody BerACT35 to monitor CD134 expression on feline cells (22, 26, 48), demonstrating that fCD134 is expressed on IL-2-dependent T cells in vitro (48) and on $CD4^+$ $CD25^+$ T-regulatory cells in vivo (26). However, recent studies have asserted that the BerACT35 antibody is human CD134 specific and does not recognize feline CD134 (12, 13). Given that the ability to monitor the surface expression of CD134 is critical to the interpretation of assays of receptor function, and in order to resolve these discordant findings, we examined the binding of BerACT35 to feline, human, and murine cells stably transduced with retroviral vectors bearing feline CD134 (Fig. 1). Feline CD134-expressing feline cells (MCC; Fig. 1A), human cells (NP2; Fig. 1B), and murine cells (NSO; Fig. 1C) displayed strong, specific binding of BerACT35 following stable transduction with the feline CD134 expression vector. To further confirm the specificity of the interaction, MCC cells were stably transduced with a retroviral vector bearing a feline CD134–C-terminal EGFP fusion (Fig. 1D). The majority of BerACT35 reactivity (CD134-PE; 74.3%) was directly proportional to the intensity of CD134-EGFP expression. Finally, BerACT35 reacted with CrFK cells following either transient transfection (Fig. 1E; 5.7% of cells were double positive for CD134-EGFP and BerACT35-PE) or stable transduction (Fig. 1F; 55.6% of cells were double positive for CD134-EGFP and BerACT35-PE) with feline CD134-EGFP expression vectors. These data are consistent with BerACT35 recognizing feline CD134 irrespective of the cellular target and means of ectopic expression.

Given that BerACT35 binds specifically to feline CD134, we examined the effect of infection with subtype A, B, and C viruses on CD134 expression on IL-2-dependent feline T cells (MYA-1 cells) (33). MYA-1 cells were infected with the GL8 (24), B2542 (15), and CPGammer (16) strains of FIV (subtypes A, B, and C, respectively) and monitored by flow cytometry for the expression of CD134 and CXCR4. In parallel, supernatants were collected to measure RT activity. FIV infection resulted

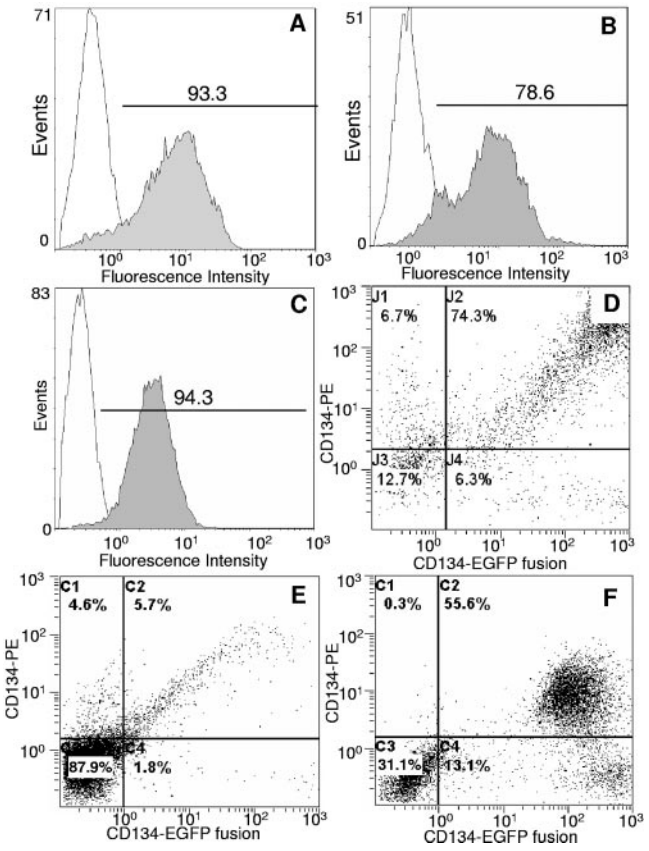

FIG. 1. BerACT35 recognizes feline CD134. BerACT35 reactivity was assessed by flow cytometry on MCC (feline) (A), NP2 (human) (B), and NSO (murine) (C) cells stably transduced with either fCD134.pDONAI (filled histograms) or pDONAI vector only (open histograms). Reactivity was also assessed on MCC (D) and CrFK (F) cells stably transduced with and CrFK cells (E) transiently transfected with fCD134.EGFP.pDONAI.

in a sharp decline in CD134 expression within 2 days postinfection (Fig. 2B, C, and D), irrespective of the viral subtype. The reduction in CD134 expression mirrored a rise in RT production and persisted for the duration of the time course. In contrast, CD134 expression remained relatively stable on uninfected control cells (Fig. 2A). Similar findings were observed with CXCR4 expression, i.e., a sharp decline in CXCR4 expression (Fig. 2G, H, and I) mirrored a concomitant rise in RT production. However, the reduction in CXCR4 expression did not persist, with expression levels recovering to control levels by the end of the time course. These results would be consistent with the expansion of a CD134-negative and FIV-resistant population of cells. To further address the specificity of the fall in CD134 expression in FIV-infected cells, MYA-1 cells were infected with the nondomestic cat lentiviruses puma lentivirus (PLV) and lion lentivirus (LLV). There was no reduction in CD134 expression in either PLV (Fig. 2C)- or LLV (Fig. 2F)-infected cells; indeed, PLV infection resulted in an increase in expression of both CXCR4 (Fig. 2I) and CD134, suggesting an increase in the activation state of the T cells (both CXCR4 and CD134 are up-regulated on activated T cells). The data suggest that the reduction in CD134 expression following FIV infection is specific to the domestic cat virus.

Expression of CD134 chimeras. In order to discern whether diverse isolates of FIV interacted with CD134 in a similar way, we mapped the functional determinants of receptor activity on feline CD134, using a series of chimeras generated between human and feline CD134 molecules (previously, we had demonstrated that human CD134 did not support infection with FIV [48]). Chimeras were designed based on the predicted three-dimensional structure of feline CD134 generated by submitting the feline CD134 amino acid sequence translation to the Swiss-Prot server in first-approach mode. The predicted structure of fCD134 was compared with those of the tumor necrosis factor receptor (2) and herpes simplex virus entry mediator (HveA) (6, 8, 9). Given the distribution of divergent amino acid residues between feline and human CD134, the most likely site for interaction between FIV Env and CD134 was predicted to lie in the first cysteine-rich domain (CRD1), analogous to the binding site for HSV gD on HveA (6, 8, 9). Chimeras were therefore designed such that the junctions were located in regions of sequence conservation between the human and feline CD134 translations and focused primarily on the first and second CRDs (Fig. 3). The CRD definitions are as described previously (31) and correspond to the molecular architectures A1B2 (CRD1 and CRD2) and A1B1 (CRD3), as detailed previously (5). The nucleic acid sequence of each chimera in the retroviral vector was confirmed prior to stable transduction of the target cell line (MCC). The surface expression of each chimera was assessed by using the BerACT35 antibody. Of the seven constructs, five were stably expressed on the cell surface in a form that was recognized by BerACT35, namely, FFF, FFH, FHF, FHH, and HHF (Fig. 4). Two chimeras (HFF and HFH) appeared to be poorly expressed at the cell surface, and moreover, the cell lines bearing these molecules took longer to expand following G418 selection, perhaps indicating an inhibitory effect on cell growth. To ascertain whether the HFF and HFH chimeras were being expressed, we generated a second series of cell lines in which the C termini of the CD134 constructs were tagged with EGFP. Transduction of MCC cells with the GFP-tagged CD134 constructs indicated that the constructs were indeed being expressed but that expression was not detected at the cell surface by BerACT35 (data not shown), indicating that either the molecule did not reach the surfaces of the cells or the BerACT35 epitope had been disrupted in these cells.

MCC cells transduced with the CD134 chimeras were infected with HIV (FIV) pseudotypes carrying a luciferase marker gene [HIV(FIV)-luc] and bearing Envs from the GL8, PPR, and TM2 strains of FIV (Fig. 5). Viral entry was detected in two of the eight cell lines, FFF (reconstructed full-length CD134) and FFH (feline $CD134_{1-85}$ × human $CD134_{86-270}$). All chimeras bearing amino acids 57 to 85 of human CD134 were rendered nonfunctional as viral receptors, despite encoding CD134 molecules that were stably expressed on the surfaces of the transduced cells and could be detected with BerACT35. Thus, the FFH chimera was a functional receptor, whereas FHH was not. These data indicated that the region spanning amino acids 1 to 86 conferred FIV receptor activity on feline CD134. Amino acids 1 to 86 comprise CRD1 and part of CRD2 and encompass the region known to form the binding domain for gD on HveA. To further define the region that conferred functional receptor activity on CD134, we prepared two additional chi-

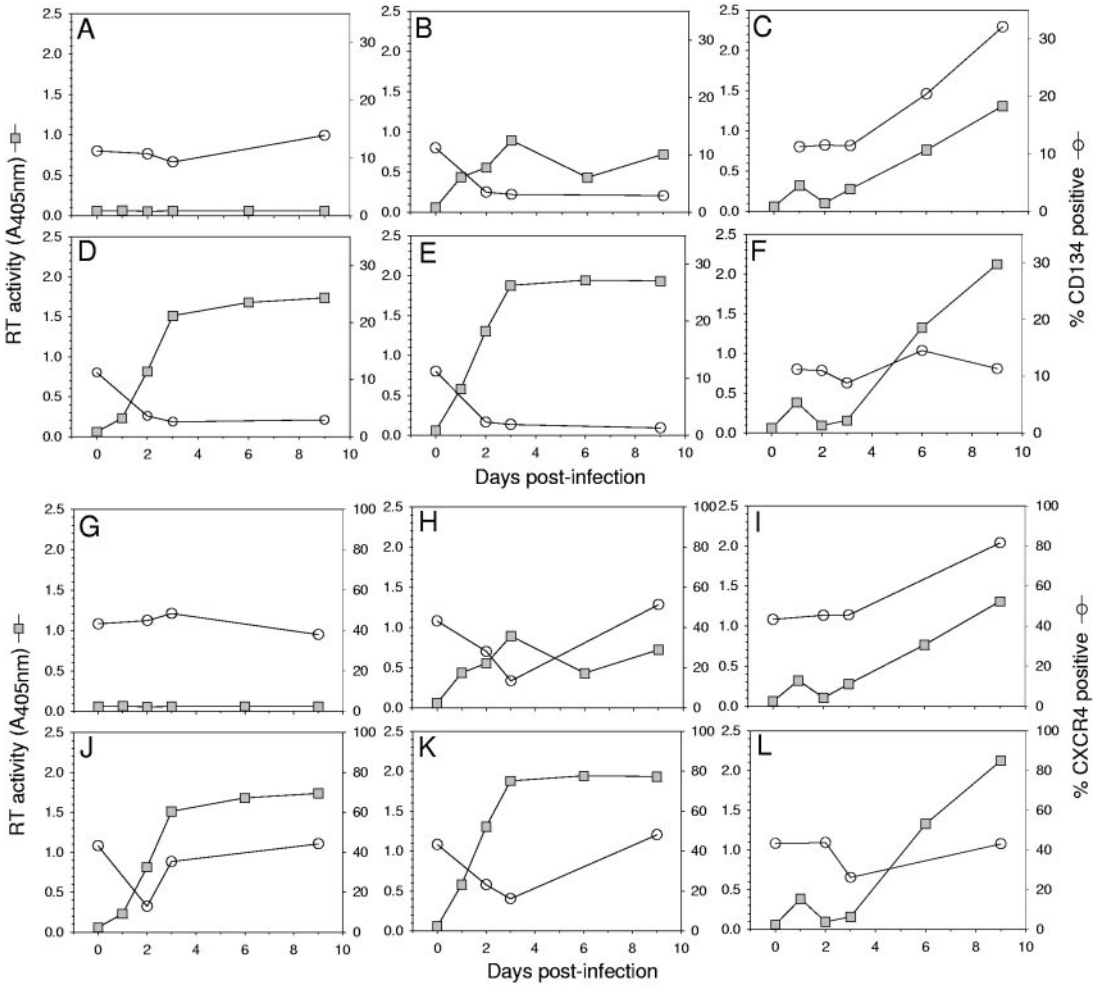

FIG. 2. Reduced surface expression of CD134 and CXCR4 on FIV-infected cells. The surface expression of CD134 (A to F) and CXCR4 (G to L) was monitored by flow cytometry using anti-CD134 (BerACT35) and anti-CXCR4 (RND44701) with IL-2-dependent T cells infected with FIV strain GL8 (B and H), B2542 (D and J), or CPGammer (E and K), PLV (C and I), or LLV (F and L) or mock infected (A and G). Percentages of positive cells were calculated relative to an isotype-matched control. Supernatants were collected in parallel, and RT activities were estimated by a nonisotopic assay, with the results expressed as the absorbance at 405 nm.

meras, exchanging amino acids 47 to 56 and amino acids 65 to 85 between chimeras FFH and FHH, generating chimeras F(FH)H and (FH)FH (Fig. 3). MCC cells were transduced with retroviral vectors bearing the novel chimeras, stably selected (cell surface expression of the chimeras was confirmed using BerACT35 [not shown]), and challenged with HIV(FIV)-luc pseudotypes bearing the GL8, PPR, and TM2 Envs (Fig. 6). The F(FH)H chimera, which effectively contained CRD1 of feline CD134 in the context of human CD134, almost completely lost the ability to act as a functional receptor for infection mediated by the GL8 or TM2 Env (Fig. 6A and C; 0.0% and 0.4% that of the FFF control). In contrast, infection mediated by PPR Env was supported with a reduced efficiency (Fig. 6B; 3.8% that of the FFF control). The (FH)FH chimera was more effective as a functional receptor, supporting viral entry mediated by the GL8, PPR, and TM2 strains (16.5, 39.3, and 21.4% that of the FFF control). It has been reported that substitution of the region defined as CRD1 from feline CD134 for human CD134 [equivalent to the F(FH)H chimera] is sufficient to confer full receptor function on a single isolate of FIV (12). The data for chimeras F(FH)H and (FH)FH are consistent with CRD1 containing a major component of the receptor binding domain. However, for reconstitution of a functional receptor, additional determinants besides CRD1 are clearly required. Given that F(FH)H did not support infection with the GL8 and TM2 pseudotypes while showing reduced activity with PPR pseudotypes, a broader range of pseudotypes was investigated for usage of the chimeric receptors. Pseudotypes were prepared bearing Envs prepared from the highly pathogenic subtype C virus FIV-CPGammer (CPG41) (16), the subtype A isolates 1419 and 0827 (48), and the subtype B NCSU1 (63) and B2542 (15) strains of FIV. The CPG41 and NCSU1 (Fig. 6D and H) strains yielded similar results to those for the GL8 and TM2 strains; the (FH)FH chimera supported viral entry mediated by CPG41, 1419, 0827, and NCSU1 Envs (32, 21, 21, and 38% that of the FFF control, respectively), while F(FH)H had no or low activity (0.0, 3.6, 0.0, and 0.9% that of the FFF control, respectively). In contrast, the subtype B isolate B2542

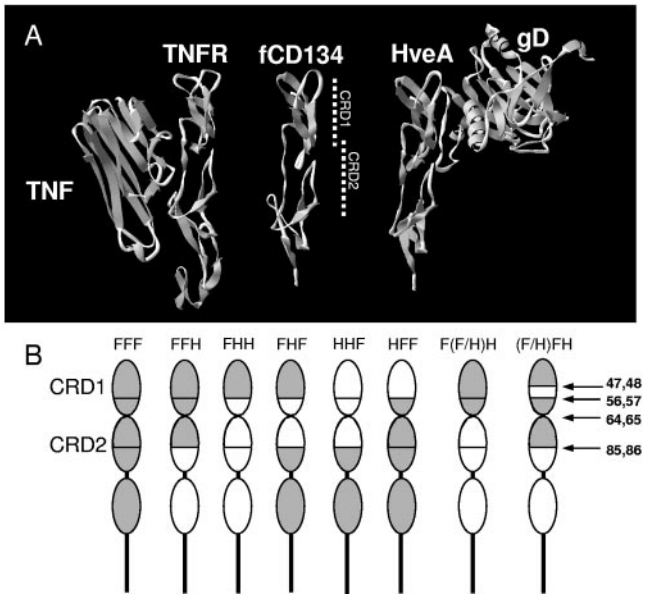

FIG. 3. Construction of feline × human CD134 chimeras. (A) Predicted three-dimensional structure of feline CD134, comparison with those of tumor necrosis factor receptor (TNFR) and HveA, and locations of CRD1 and CRD2. (B) Schematic of feline × human CD134 chimeras showing the locations of the junctions between the feline (gray) and human (white) regions. Numbered arrows indicate amino acids flanking the junctions.

(Fig. 6E) yielded findings with PPR, utilizing both the (FH)FH and F(FH)H chimeras efficiently (76% and 24% of the FFF control value). The data suggest that determinants other than CRD1 in the region spanning amino acids 65 to 85 are required to form a functional receptor for the majority of virus strains. The fact that the F(FH)H chimera showed activity with the B2542 and PPR strains may provide evidence for the utilization of distinct regions of CD134 by diverse strains of FIV.

Additional cell lines stably expressing the (FH)FH and F(FH)H constructs and based on the HeLa cell line were generated in order to assess the reproducibility of the findings with MCC cells (MCC cells are feline lymphoid cells, while HeLa cells are human epithelioid cells). The cells were then challenged with HIV(FIV)-luc pseudotypes bearing a range of Envs. The results are summarized in Table 1, with receptor function expressed as the increase in susceptibility relative to cells transduced with vector only. Strains GL8, CPG41, TM2, 0827, and NCSU1 showed a preference for (FH)FH over F(FH)H which was most marked with the FIV-CPGammer (16) (CPG41) Env, which used (FH)FH 59-fold and 269-fold more efficiently than F(FH)H in MCC and HeLa cells, respectively. The data confirm that determinants in the region spanning amino acids 65 to 85 of CD134 are required for viral entry to proceed.

We next asked whether cells expressing the (FH)FH and F(FH)H chimeras showed differences in the ability to support productive infection (Fig. 7). CLL cells expressing the chimeric receptor molecules were infected with matched doses of the cloned viruses GL8 and GL8.CPG41 (preference for FHFH) or GL8.B2542 and PPR (no preference or weak preference). Cells were infected in parallel with HIV (FIV) pseudotypes bearing the equivalent Envs. Whereas assays using viral pseudotypes measure viral entry alone, cell-free virus infection represents the sum of entry, replication, egress, and cell-to-cell spread. Therefore, we predicted that the efficiencies of the chimeras as viral receptors might be less evident in an assay for productive virus infection. HIV (FIV) pseudotypes bearing the GL8 and CPG41 Envs used the FHFH chimera with greater efficiencies than those for FFHH in CLL cells, while PPR or B2542 pseudotypes used both FHFH and FFHH efficiently (Fig. 7A), consistent with the previous findings using MCC and HeLa cells transduced with the FFHH and FHFH expression vectors (Table 1). It was notable that viral entry into the vector-only control CLL cells was ~10-fold higher than that for GL8, PPR, B2542, and CPG41. These data may suggest an upregulation of endogenous canine CD134 during the selection process; studies are currently under way to address whether this is indeed the case.

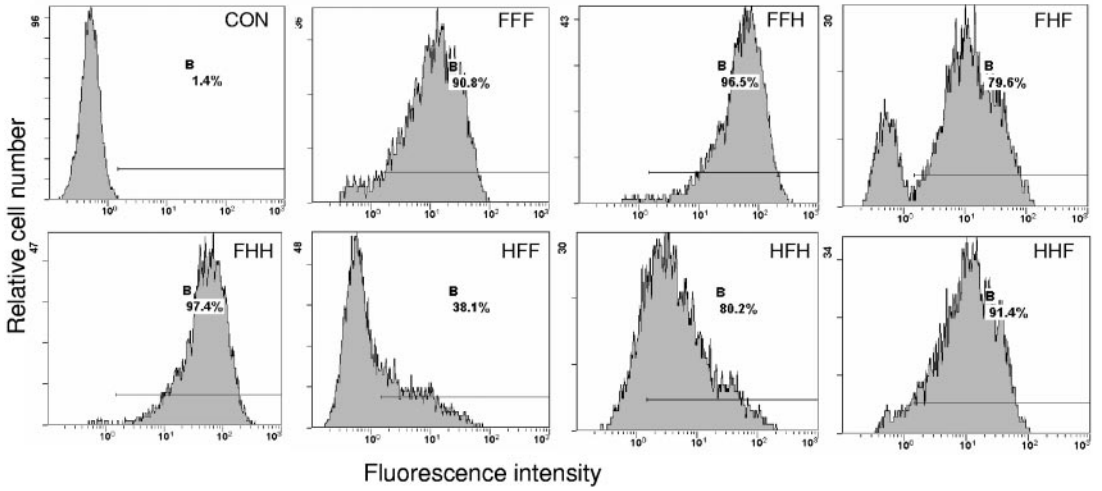

FIG. 4. Surface expression of CD134 chimeras. MCC cells were stably transduced with a retroviral vector bearing each chimera. Cell surface expression of CD134 was analyzed by flow cytometry using BerACT35. Each histogram represents 10,000 events and is representative of two analyses.

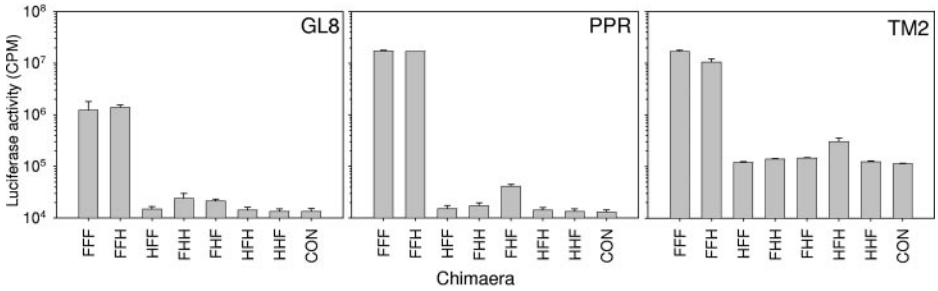

FIG. 5. Ability of CD134 chimeras to act as functional receptors for FIV. MCC cells stably transduced with a retroviral vector (pDONAI) bearing each of the chimeras or with vector only (CON) were infected with HIV (FIV) luciferase pseudotypes bearing the GL8, PPR, or TM2 Env. Luciferase activities were assayed at 72 h postinfection and are expressed as mean ($n = 3$) counts per minute (CPM) ± standard errors (SE).

Productive infection experiments recapitulated these findings in that GL8 and GL8.CPG41 replicated more efficiently in CLL-FHFH cells than in CLL-FFHH cells. GL8-B2542 and PPR did not display a preference for CLL-FHFH cells over CLL-FFHH cells; indeed, B2542 replicated more efficiently in CLL-FFHH cells than in CLL-FHFH cells (Fig. 7B), suggesting that in this system, the selectivity for the two chimeras extended to the replicative capacity.

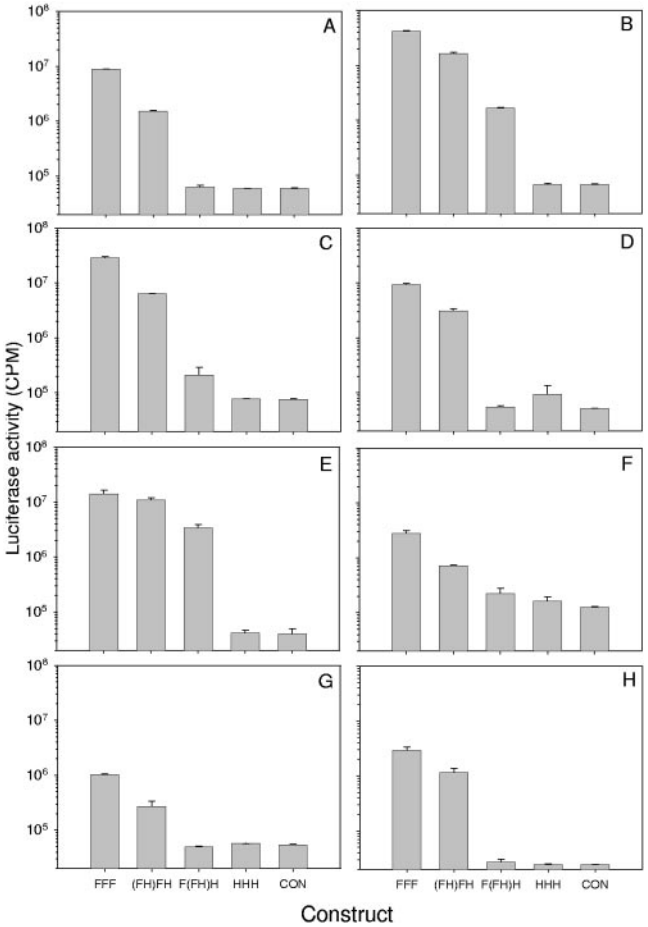

FIG. 6. Differential utilization of CD134 chimeras by FIV strains. MCC cells stably transduced with a retroviral vector (pDONAI) bearing each of the chimeras [FFF, (FH)FH, F(FH)H, and HHH] or with vector only (CON) were infected with HIV (FIV) luciferase pseudotypes bearing the GL8 (A), PPR (B), TM2 (C), CPG41 (D), B2542 (E), 1419 (F), 0827 (G), and NCSU1 (H) Envs. Luciferase activities were assayed at 72 h postinfection and are expressed as mean ($n = 3$) counts per minute (CPM) ± SE.

DISCUSSION

The virus-receptor interaction is the primary event in the viral replicative cycle, a major determinant of cell tropism, and a target for therapeutic intervention. Antibodies that block the HIV-CD4 interaction have been shown to have broad neutralizing activity (anti-CD4-binding-site antibodies), neutralizing strains of virus from diverse clades and geographical origins. In this study, we investigated the binding site for FIV Env on CD134, the cellular receptor for FIV. We showed that primary isolates of FIV can be classified into at least two groupings based on their interactions with CD134. The expression of feline CD134 CRD1 alone in the context of human CD134 is sufficient to confer nearly optimal receptor function for infection with strains such as PPR, in agreement with recent findings (12). However, pathogenic primary strains of virus, such as GL8, CPGammer, and NCSU1, require additional determinants in CRD2 for the restoration of receptor function. These data provide the first evidence for the differential utilization of feline CD134 by FIV. The extent of the binding site utilized by

TABLE 1. Comparison of CD134 chimera utilization by diverse strains of FIV

Virus	Fold increase in MCC cell susceptibility with chimera		Ratio[a]	Fold increase in HeLa cell susceptibility with chimera		Ratio[a]
	(FH)FH	F(FH)H		(FH)FH	FFHH	
GL8	25	1	25*	1,720	10	172*
PPR	609	241	2.5	4,919	2,608	1.9
TM2	82	2	41*	1,878	199	9.4*
CPG41	59	1	59*	2,690	10	269*
0425	3	5	0.6	107	95	1.1
1419	5	1	5	577	720	0.8
0827	5	1	5	459	13	35.3*
B2542	275	85	3.2	302	220	1.3
NCSU1	49	1	49*	2,802	798	3.5

[a] *, FIV Env pseudotypes displaying a preference for (FH)FH over F(FH)H. Results are typical of two separate experiments.

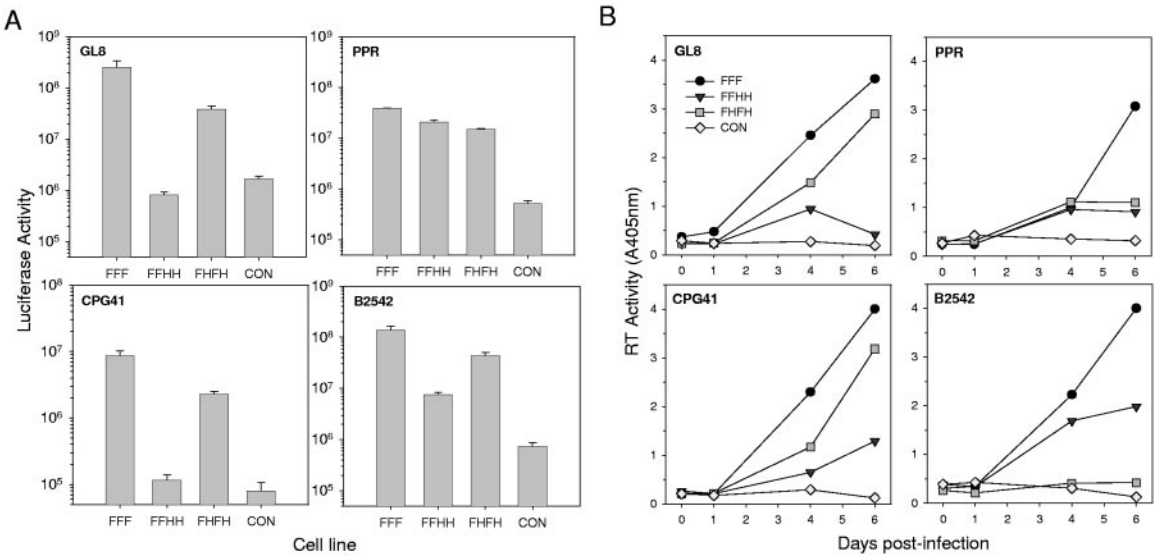

FIG. 7. Productive infection of cells expressing CD134 chimeras. (A) CLL cells stably transduced with a retroviral vector (pDONAI) bearing each of the chimeras [FFF, (FH)FH, and F(FH)H] or with vector only (CON) were infected with HIV (FIV) pseudotypes bearing the GL8, B2542, CPG41, and PPR Envs and assayed for viral entry by a luciferase assay. Luciferase activities were assayed at 72 h postinfection and are expressed as mean ($n = 3$) counts per minute (CPM) $\pm$ SE. (B) Cells were infected in parallel with matched titers of replication-competent virus from the molecular clones GL8, GL8.2542, GL8.CPG41, and PPR; supernatants were collected on days 0, 1, 4, and 6 postinfection and assayed for virus production by their nonisotopic reverse transcriptase activities (absorbance at 405 nm). Results represent the means of duplicate experiments (each time point is the mean of two replicates).

strains such as GL8, CPGammer, and NCSU1 remains to be established; the amino acid substitutions S60D, N62D, H45S, R59G, and V64K in human CD134 recreated the binding site for FIV PPR Env and restored infectivity for CrFK cells, in contrast to the F(FH)H chimera used in this study (containing all of the above amino acids), which had nearly negligible activity as a receptor for the GL8 and CPG41 Envs. The PPR strain infects CrFK cells with a low efficiency in the absence of CD134 (12), and overexpression of human CXCR4 alone is sufficient to render (CD134-negative) G355 cells permissive for productive infection with FIV PPR (14). These data may indicate either a reduced requirement for CD134 or a reduced threshold for induction of the conformational change in the PPR Env that permits a direct interaction with CXCR4. Consistent with this hypothesis, an FIV PPR SU-Fc fusion protein bound to 60% of purified resting feline peripheral blood mononuclear cells, and the majority of binding was via CXCR4 alone (13), suggesting that viruses such as PPR may have a low requirement for CD134 for binding/infection. A reduced activation threshold for envelope-mediated fusion has been implicated in the propensity for CD4-independent infection by HIV-2 (44). Given that the interaction of the GL8, CPG41, and NCSU1 Envs would appear to be more complex than that of PPR Env, extending beyond CRD1, our studies raise the possibility that infection by such viruses may well be modulated by the natural ligand for feline CD134 (CD134L or OX40 ligand).

The interaction between HIV and CD4 induces a conformational change in gp120 that results in the exposure of the chemokine receptor binding site; this conformational change is marked by the exposure of epitopes for CD4-induced antibodies such as 17b and 48d (30, 50, 51, 53, 62). Primary strains of HIV become more sensitive to soluble CD4 (sCD4) with prolonged culture (1, 34, 35). With the discovery of the coreceptors CXCR4 and CCR5, it became apparent that X4 variants of HIV were more sensitive to surface CD4 concentration than R5 strains of virus (29). Subsequently, the affinity of the interaction between Env and CD4 was shown to be critical to the process of adaptation to cell culture (42), and primary and laboratory-adapted strains of HIV were shown to display differential requirements for levels of both CD4 and CXCR4 (56). By analogy with HIV, our data may indicate that some primary strains of FIV require a more complex interaction with CD134 than others for infection to proceed. Although reagents with which the interaction between these primary Envs and CD134 may be dissected are not currently available, future studies should investigate whether the complexity of the interaction between Env and CD134 for strains such as GL8 is reflected in an increased (or decreased) binding affinity for CD134. An increased affinity for CD4 has been shown to accompany laboratory adaptation of HIV (42). One of the FIV strains (PPR) able to utilize the fCD134 CDR1-only chimera has been shown previously to infect CD134-negative feline cells following overexpression of human CXCR4 (14). Although human CXCR4 does have an intrinsically higher activity as a coreceptor for FIV than does feline CXCR4 (58), these data may indicate that strains such as PPR, with a less stringent interaction with CD134, may be more readily adapted for culture in CD134-negative cell lines. Moreover, the emergence of such strains in vivo may accompany a broadening of the viral cell tropism, as observed with the shift from acute to chronic FIV infection (11, 20). The link between the nature of the Env-CD134 interaction and the broadening of viral cell tropism with disease progression will be an important avenue of future research.

The surface expression of feline CD134 and the feline × human CD134 chimeras was monitored using the anti-human CD134 antibody BerACT35, and yet previous studies (12, 13) have suggested that this antibody is human CD134 specific. Given the importance of this reagent to the study of the virus-receptor interaction, it is crucial that these discordant observations be reconciled. BerACT35 has been used to monitor feline CD134 expression in vitro and in vivo (22, 26, 48). Furthermore, we have shown that surface CD134 expression can be evaluated using BerACT35 following stable transduction of the adherent cell lines HeLa, NP2, and AH927 or the suspension cell lines NSO, MCC, and 3201 with retroviral vectors bearing feline CD134, suggesting that in this system, feline CD134 is expressed in the same conformation as the endogenously expressed molecule. The studies in which BerACT35 failed to recognize feline CD134 (12, 13) were performed using transient retroviral transduction of CrFK cells; it is possible that in such an expression system, feline CD134 may be expressed in an antigenic conformation distinct from that present in vivo (26). The significance of these distinct antigenic conformations for FIV receptor function remains to be established; however, given that Ber ACT35 recognizes feline CD134 in vivo, this antigenic conformation is likely to be biologically relevant.

CD4-independent strains of HIV are particularly sensitive to neutralizing antibodies (18, 27, 28, 43) and thus may be more likely to arise where the host humoral immune response is failing or in immunoprivileged sites (reviewed in reference 4). CD134-independent strains of FIV are readily neutralized by sera from infected animals, most markedly when the assays are performed on CD134-negative, CXCR4-positive cells (36). Similarly, neutralizing antibodies against CD4-independent strains of HIV/SIV are detected more readily when the assays are based on CD4-negative cells (43, 54). Many of the prototypic strains of FIV were either isolated from animals with clinical signs of AIDS (39, 41) or have subsequently undergone prolonged culture in vitro (49, 52, 57), with the consequence of a degree of selection for neutralization-sensitive strains of virus with a propensity for adaptation to CD134-independent infection. Given that the in vivo cell tropism of FIV would appear to expand with disease progression (11, 20), it is likely that the strains of virus present in animals displaying clinical signs differ from those present in the early/acute phase of infection, analogous to the shift in cell tropism observed with the emergence of X4 strains of HIV with the progression to AIDS (10). Future studies should address whether strains of FIV present in the early acute phase of infection differ in their in vitro cell tropism and receptor usage from those isolated from animals displaying clinical signs. Vaccine efforts may then be targeted against the strains of virus that are most likely to be transmitted between animals.

ACKNOWLEDGMENTS

We thank Ed Hoover, Sue Vandewoude, Takayuki Miyazawa, Wayne Tompkins, Yasuhiro Takeuchi, and Yasuhiro Ikeda for the provision of reagents and for helpful discussions.

This work was supported by Public Health Service grant AI049765 from the National Institute of Allergy and Infectious Diseases and by the Medical Research Council, United Kingdom.

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